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Conformational studies of hexapeptides containing two dehydroamino acid residues in positions 3 and 5 in peptide chain

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ABSTRACT

Synthesis and structural studies of hexapeptides containing two dehydroamino acid residues in positions 3 and 5 in a peptide chain were performed. All the investigated peptides adopted bent conformations, stabilized by intramolecular hydrogen bonding, and could exist as two different conformers in solution. Only in the case of the peptide containing ΔAla residues, expected 3_{10}-helical conformation was found.

1. Introduction

Rational interpretation of the results of biological tests and rational designing of new compounds require the detailed knowledge about spatial structure–activity relationship. One of the main streams in drug discovery has focused attention on the synthesis of small mimetics of potent therapeutic peptides, both natural and synthetic ones. For this reason, the search for new factors, which could be used as modifiers of peptide chain conformation, has become increasingly important.

Introduction of dehydroamino acid residues into peptide chain has been found to influence the three dimensional structure of both main chain and side chain dramatically, due to the presence of $C^{\alpha}=C^{\beta}$ double bond [1]. For example, dehydroalanine adopts a roughly planar conformation with *trans* orientation for the ψ and φ torsions and induces an inverse γ-turn in the preceding residue [2]. (*Z*)-Dehydrophenylalanine exerts a β-turn conformation in short peptides [3] and 3_{10}-helical conformation in the case of peptides with a longer main chain [4–6]. For dehydropeptides containing ΔX-Y-ΔX motif, the most favorable conformation was 3_{10}-helix [7–10]. In the case of peptides containing two dehydrophenylalanines separated by more than one saturated residue, besides 3_{10}-helix, α-helix could also be found. This tendency is stronger while the saturated sequence between two dehydroamino acids is longer [11]. If 3_{10}-helix conformation is stabilized by intramolecular hydrogen bond of $(i+3) \rightarrow I$ type, and α-helix by $(i+4) \rightarrow i$, this mixed conformation is stabilized by both these types of bonds [12]. Moreover, folded conformation present in relatively nonpolar solvent ($CHCl_3$) is destabilized in DMSO and peptides adopt largely extended conformation in this solvent [13]. Taken together, literature data suggested that dehydroamino acid residues exert a powerful conformational influence, independent on other constraints. Thus, introduction of dehydroamino acid residues into bioactive peptide sequences has become a useful tool to study structure–function relationship and to provide new analogues of enhanced activity.

Cathepsin C (dipeptidyl dipeptidase I; EC 3.4.14.1) belongs to papain family of proteases [14] and sequentially removes dipeptides from the free N-termini of proteins and peptides. It has a broad substrate specificity being able to hydrolyse out nearly every possible dipeptide unit, with the exception of those containing basic amino acids (Arg or Lys) at N-terminal position or Pro on either side of the scissile bond. It is also quite unusual that it has a requirement for the presence of halide ions for its activity. The main function of cathepsin C is protein degradation in lysosymes, but it is also found to participate in the activation of cytotoxic T lymphocytes and natural killer cells (granzymes A and B), mast cells (tryptase and chymase), and neutrophils (cathepsin G and elastase) by removing their N-terminal activation dipeptides [15–17]. Loss of function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis [18]. Since dehydroamino acids are quite reactive and various thiol nucleophiles are known to add to their double bonds [15–18], we speculated that

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short dehydropeptide mimetics of artificial substrates of cathepsin C might act as alkylating inhibitors of the enzyme. However, our former studies of tetra- and pentapeptides containing ΔAla and Δ^{Z}Phe residues showed that they acted only as substrates or weak inhibitors of cathepsin C [19–21]. For this reason, in the case of longer chains, such as the hexapeptides presented in this paper, we focused only on structural investigations.

Conformational studies on pentapeptides containing two ΔPhe residues (*Z* and *E* isomers) in positions 2 and 4 in the peptide chain showed that all the investigated peptides adopted bent conformation and majority of them could exist as two different low-energetic conformers in solution [21]. In the case of hexapeptides containing two dehydroamino acid residues located in positions 2 and 5, only for the peptide with free N-termini, rigid 3_{10}-helical conformation was observed. For the rest of the examined peptides extended and "zig-zag" conformers were predominant [22]. The next step of our investigations, presented in this paper, is devoted to the conformational studies of hexapeptides, where dehydroamino acids are isolated only by one saturated residue and located in positions 3 and 5 in peptide chain (see Fig. 1).

2. Results and discussion

2.1. Synthesis

Peptides were obtained analogously to previously described procedure [21,22], via 1 + 5 approach, by reacting Boc-GlyOH with Gly-X-Gly-X-Phe-pNA in acetonitrile using TBTU as a coupling reagent. Products were purified by repetitive precipitation from *iso*propanol by addition of hexane. They were of amorphous state. Yields of Boc-protected peptides were: 71% (peptide no. 2), 76% (peptide no. 4), 83% (peptide no. 5) and 74% (Boc-Gly-Gly-ΔAla-Gly-ΔAla-Phe-pNA). Blocking groups were removed with trifluoroacetic acid according to standard procedure [21], yielding peptides no. 1 and no. 3 with 96–99% yield.

R-Gly-Gly-X-Gly-X-Phe-pNA

Peptide	R-	-X-
1	H	ΔAla
2	Boc	Δ^{Z}Phe
3	H	Δ^{Z}Phe
4	Boc	Δ^{E}Phe
5	Boc	Phe

Fig. 1. Investigated peptides.

2.2. Structural and conformational studies

NMR and CD studies were done in order to determine the structures and conformational preferences of dehydropeptides shown in Fig. 1.

2.3. CD spectroscopy

CD measurements were performed in two solvents (chloroform and methanol) for the region down to 200 nm because at shorter wavelengths there are overlapping contributions of the peptide, aromatic and unsaturated chromophores, which makes the analysis very complex. In the near-UV region, Cotton effects associated with ΔPhe and pNA groups were observed. This is strongly dependent on the peptide conformation and therefore very useful in this kind of conformational studies [23].

Analysis of CD spectra obtained in methanolic solution (Fig. 2a) shows that the most unordered conformation is observed in the case of saturated peptide no5. On the other hand, strong negative band detected at 220 nm for peptide no4 (Boc-Gly-Gly-Δ^{E}Phe-Gly-Δ^{E}Phe-Phe-pNA) and strong positive band at value 305 nm for peptide no3 suggest that compounds which possess ΔPhe in the chain adopt a more ordered conformation. Comparison of CD spectra obtained for chloroform solutions of two structural isomers (Fig. 2b) shows that the peptide containing *Z*-isomers of phenylalanine adopts a more ordered conformation. This result is surprising in the sense of our former studies [20]. However, during the storage of this class of peptides containing Δ^{E}Phe, we observed tendency for them to isomerise to *Z*-isomer, which could influence the obtained results. Due to low solubility in $CHCl_3$ it was not possible to measure the CD spectra of the rest of the investigated peptides.

2.4. NMR spectroscopy

In order to find some evidence of the presence of intramolecular hydrogen bonds in the studied peptides, we have performed measurements of the influence of temperature on the chemical shifts of their amide protons. The experiments were done in DMSO continuously increasing temperature. The obtained results are presented in Table 1. The main parameter indicating the presence of hydrogen bond is the value of $d\delta/dT$ [ppm/K]. It is well established that the existence of hydrogen bond is reflected in the value of this coefficient being lower than 0.004 [ppm/K]. Results obtained from

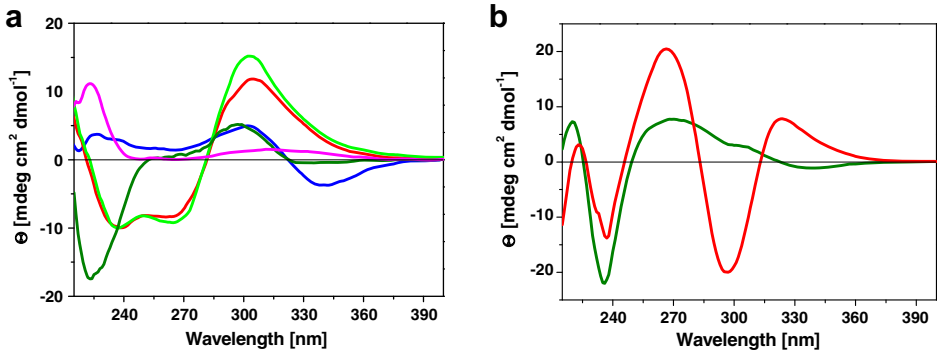

Fig. 2. CD spectra obtained for the investigated peptides (a) in methanol – peptide no. 1 (blue), peptide no. 2 (red), peptide no. 3 (green), peptide no. 4 (olive), peptide no. 5 (magenta), (b) in chloroform – peptide no. 2 (red), peptide no. 4 (olive).

Table 1
Temperature dependence of chemical shifts of amide protons of the investigated peptides in DMSO solution (no. 2a – $CDCl_3$ solution) – temperature coefficient $d\delta/dT$ (ppm K^{-1})

Peptide	Gly[1]	Gly[2]	ΔPhe[3]/ΔAla[3]	Gly[4]	ΔPhe[5]/ΔAla[5]	Phe[6]	HN pNA
No. 1	**0.0021**	0.0043	[a]	0.0051	0.0066	0.0055	0.0063
No. 2	0.0067	0.0050	**0.0007**	0.0051	0.0069	0.0047	**0.0032**
No. 2a	[a]	0.0068	**0.0036**	0.0047	0.0055	[a]	**0.0029**
No. 3	**0.0021**	0.0045	0.0052	0.0055	0.0067	0.0047	**0.0030**
No. 4	0.0069	**0.0004**	0.0060	0.0046	0.0058	0.082	**0.0028**

[a] Overlapping of the signals.

the experiments (see Table 1) showed that in every investigated peptide, conformation could be stabilised by intramolecular hydrogen bonding. Except in peptide no. 1 (H_2N-Gly-Gly-ΔAla-Gly-ΔAla-Phe-pNa), in all other cases amide proton of *p*-nitroanilide moiety could be involved in such an interaction. This tendency was observed also in the case of change of solvent from DMSO to $CDCl_3$ (see results for peptide 2a in Table 1). Contrary to hexapeptides containing two dehydroamino acids isolated by two saturated residues [22], the influence of free N-termini on the ability to create hydrogen bonding is not observed.

The most informative data about conformational preferences of the investigated peptides were obtained from NOESY and ROESY experiments. Based on the distance constraints and contacts obtained from these spectra optimization of 50 of the most stable conformers was calculated by using X-PLOR [24] programme (Figs. 3–8). The average values obtained for dihedral angles are presented in Table 2.

The results of spectroscopic studies and calculations suggest that all the investigated dehydropeptides could exist in two possible conformations (of lower and higher energy) in solution. In contrast to their saturated analogue (peptide no. 5 – see Fig. 7), they adopt ordered, stable conformation in solution. In the case of peptide no. 1 (H_2N-Gly-Gly-ΔAla-Gly-ΔAla-Phe-pNA) values of the dihedral angles (Table 2) are typical for 3_{10}-helical conformation, which is in good agreement with literature data [11,13]. For the rest of the studied compounds, obtained values of dihedral angles are not similar. Comparison of two analogous peptides no. 2 and no. 4, where the only difference in the structure is the type of dehydrophenylalanine isomer, confirmed the strong influence of this factor on conformational preferences of main chain – peptides adopt different conformations with totally different values of dihedral angles. What is also interesting is that peptide no. 4 (Boc-Gly-Gly-Δ^EPhe-Gly-Δ^EPhe-Phe-pNA) could exist in two almost identical conformations, where the only differences are observed for the values of φ [4], Ψ [4], φ [5] and ψ [5].

A very interesting result has been obtained after the comparison of conformational preferences of peptide no. 2 (Boc-Gly-Gly-Δ^ZPhe-Gly-Δ^ZPhe-Phe-pNA) in DMSO and $CDCl_3$ solutions (see Fig. 8). The conformations of the main chains are similar in both the cases (Fig. 8c), the difference could be observed for the location of phenyl rings – in the case of a non-polar solvent they create dome kind of shield over the more polar parts of the peptide (Fig. 8b).

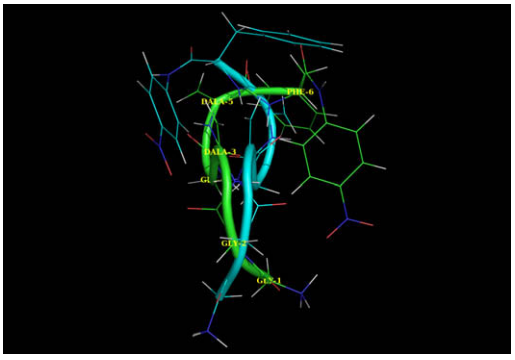

Fig. 3. Superposition of the most stable conformation of peptide no. 1 (H_2N-Gly-Gly-ΔAla-Gly-ΔAla-Phe-pNA) proposed based on X-PLOR calculation.

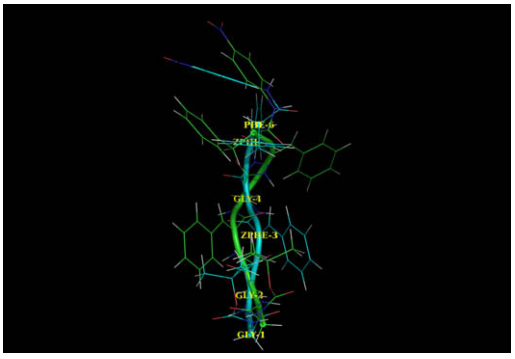

Fig. 4. Superposition of the most stable conformation of peptide no. 2 (Boc-Gly-Gly-Δ^ZPhe-Gly-Δ^ZPhe-Phe-pNA) proposed based on X-PLOR calculation.

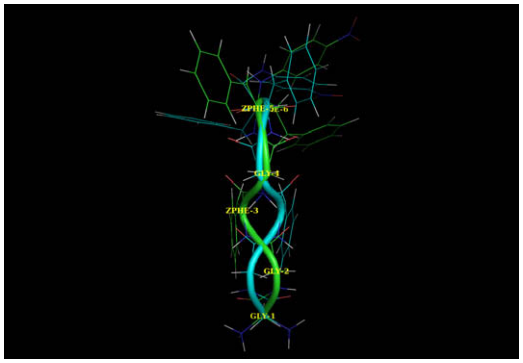

Fig. 5. Superposition of the most stable conformations of peptide no. 3 (H_2N-Gly-Gly-Δ^ZPhe-Gly-Δ^ZPhe-Phe-pNA) proposed based on X-PLOR calculation.

3. Conclusions

All the investigated peptides adopted bent conformation, which is stabilized by intramolecular hydrogen bonding, and they could exist as two different conformers in solution. Only in the case of peptide containing two ΔAla residues expected 3_{10}-helical conformation was found, whereas for the rest of the dehydropeptides various bent conformations were observed. In contrast to the previously studied hexapeptides, containing dehydroamino acids separated by two saturated residues, in this group of compounds the influence of free N-termini on the conformation of peptide was not significant.

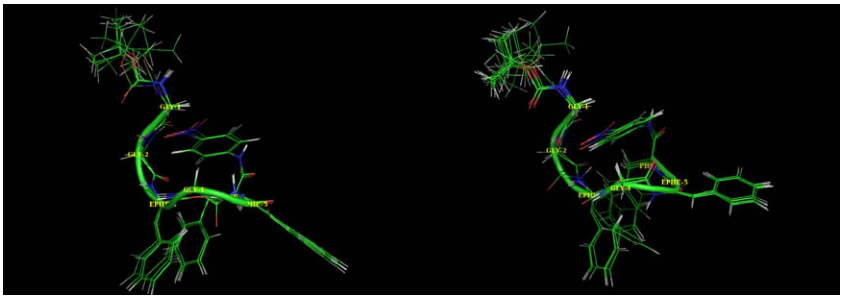

Fig. 6. The most stable conformations obtained for peptide no. 4 (Boc-Gly-Gly-Δ^{E}Phe-Gly-Δ^{E}Phe-Phe-pNA), conformers **4a** (left) and **4b** (right) proposed based on X-PLOR calculation.

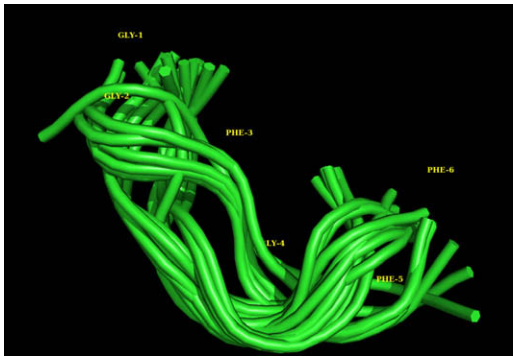

Fig. 7. The most stable conformations obtained for peptide no. 5 (Boc-Gly-Gly-Phe-Gly-Phe-Phe-pNA) proposed based on X-PLOR calculation.

4. Experimental

4.1. General

Materials were obtained from commercial suppliers (Sigma–Aldrich, Fluka, Merck) and used without purification unless otherwise stated. Column chromatography was performed on silica gel H60 (70–230 mesh).

4.2. NMR spectroscopy

NMR spectra were recorded on Bruker Avance DRX300, Bruker AMX600 and Bruker AMX400 instruments in deuterated DMSO, chemical shifts are given in relation to $SiMe_4$. In all the cases 15 mM peptide solutions were prepared. NMR spectral signal assignment and integration were carried out with SPARKY [25]. Where it was possible, the separation between two geminal protons in $-CH_2-$ group was used as a reference in distance calculations, in the other cases to calculate distance were taken from the intensity of the cross and diagonal peaks by using the correction factor [26]. Conformational calculations were carried out with X-PLOR [24]. In view of the investigated peptides containing unnatural aminoacid residues, it was necessary to modify the topology file available in distribution. To build the required topology for Δ^{Z}Phe and Δ^{E}Phe, the available topology for Phe was used. In addition, a big force constant warranting planarity of the dehydroaminoacid residues was added.

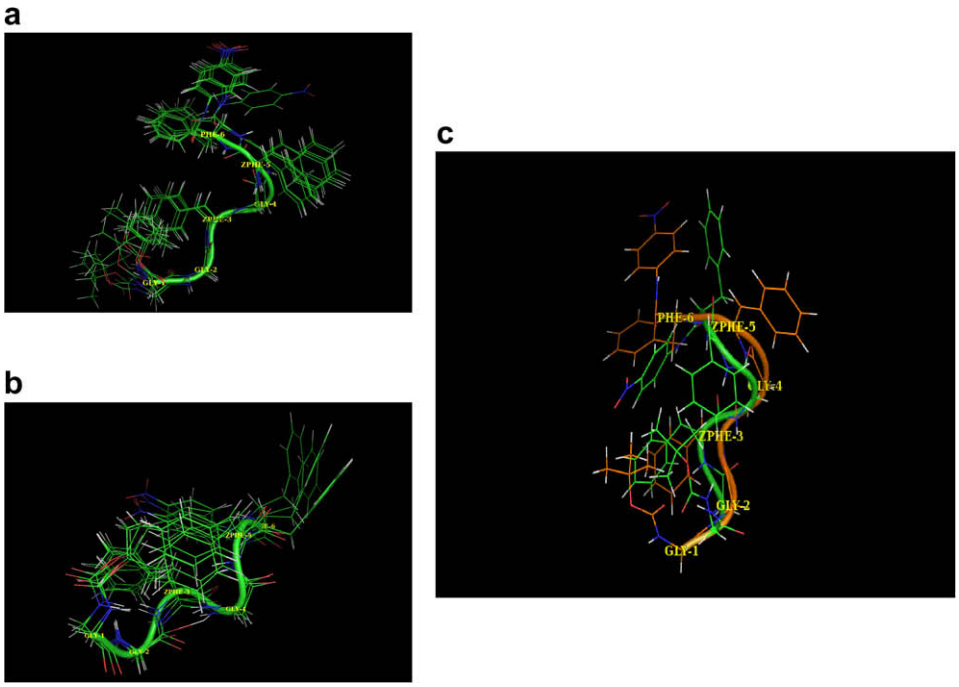

Fig. 8. Dependence of solvent on the conformation of Boc-Gly-Gly-Δ^{Z}Phe-Gly-Δ^{Z}Phe-Phe-pNA. (a) DMSO solution, (b) $CDCl_3$ solution, (c) superposition of conformations in both solvents.

Table 2
Average values of dihedral angle [°] obtained on basis of X-PLOR calculations (2a′ and 2b′–conformers observed in $CDCl_3$ solution)

Pep	φ[1]	ψ[1]	φ[2]	ψ[2]	φ[3]	ψ[3]	Φ[4]	ψ[4]	φ[5]	ψ[5]	φ[6]	ψ[6]
1a	–	155.39	162.41	−95.47	−57.47	−20.33	67.51	−63.03	−72.50	53.07	54.33	93.92
1b	–	64.51	−135.6	100.34	55.49	24.05	−60.98	74.89	58.38	−6.11	61.24	99.47
2a	105.12	89.40	76.05	−102.0	67.35	93.21	−75.56	43.30	32.42	34.07	−91.37	91.03
2b	102.15	−112.4	−86.80	125.30	−72.88	−94.77	73.14	−20.86	−2.31	110.45	−84.95	89.33
2a'	162.9	−53.6	−125.7	−60.2	7.3	−44.1	66.4	30.3	39.1	53.1	−100.9	54.4
2b'	−133.7	44.1	121.5	44.9	23.8	42.8	−61.1	−36.9	−33.7	−70.4	−37.3	−9.5
3a	–	165.85	−52.97	87.39	−78.13	47.46	−75.23	14.76	11.18	87.69	−167.03	93.97
3b	–	−172.87	89.70	−83.78	76.83	−46.83	70.73	−82.80	−74.84	−99.51	68.28	86.13
4a	51.71	−95.36	81.35	72.86	99.09	77.31	−43.80	113.48	82.47	−26.18	−68.27	−40.73
4b	51.20	−98.13	79.63	74.21	93.10	81.54	−28.40	−48.75	−88.73	33.35	−113.20	−43.91
5	7.27	−4.55	−36.28	20.11	61.51	158.42	42.50	−12.52	36.82	77.55	136.36	95.51

4.2. CD spectroscopy

CD spectra were recorded on Jasco J-715 spectropolarimeter, at room temperature. Spectra were measured in MeOH and $CHCl_3$. Pathlength of 1 mm was used. Concentrations of the solutions were ~0.3 mg/mL. Each spectrum represents the average of at least four scans. The data are presented as molar ellipticity [θ].

4.3. Peptide no. 1

^{1}H NMR (DMSO, TMS): 2.97 ppm (HB1, Phe[6]); 3.12 ppm (HB2, Phe[6]); 3.63 ppm (HA, Gly[1]); 3.84 ppm (HA, Gly[4]); 3.88 ppm (HA, Gly[2]); 4.69 ppm (HA, Phe[6]); 7.19–726 ppm (aromatic Phe[6]+HNΔAla[3]+HB#ΔAla[3]); 7.76 ppm (HB#, ΔAla[5]); 7.89 ppm (HD#pNA); 8.10 ppm (HNGly[1]); 8.22 ppm (HE#pNA); 8.38 ppm (HNGly[4]); 8.46 ppm (HN,Phe[6]); 8.71 ppm (HNGly[2]); 10.72 ppm (HNΔAla[5]); 10.75 ppm (HN,pNA); ^{13}C NMR (DMSO, TMS): 37.1 ppm (C^{β}Phe[6]); 40.1 ppm (C^{α}Gly[1]); 41.9 ppm (C^{α}Gly[2]); 42.1 ppm (C^{α}Gly[4]); 55.2 ppm (C^{α}Phe[6]); 119.0 ppm (C^{δ}#pNA); 119.1 ppm ($C^{\beta\Delta}$Ala[3], ΔAla[5]); 124.8 ppm (C^{ε}#pNA); 126.6–137.1 ppm (aromatic ring, Phe[6]); 142.8 ppm (C^{ζ}pNA); 144.7 ppm (C^{γ}pNA); 166.6 ppm (COGly[1]); 168.9 ppm (CO Gly[2]); 170.6 ppm (COPhe[6]). Elemental analysis for $C_{27}H_{30}N_8O_8$ calculated: 54.54% C, 5.08% H and 15.08% N, found: 52.25% C, 4.31% H and 13.10% N.

4.4. Peptide no. 2

Obtained in % yield by crystallization from ethyl acetate–hexane mixture. Mp 197–200 °C. ^{1}H NMR (DMSO, TMS): 1.37 ppm (CH3Boc); 3.14 ppm ($H^{\beta 1}$Phe[6]); 3.59 ppm ($H^{\alpha\#}$Gly[1]); 3.92 ppm ($H^{\alpha\#}$Gly[2]); 4.02 ppm ($H^{\alpha\#}$Gly[4]); 4.72 ppm (H^{α}Phe[6]); 6.99 ppm ($H^{\beta\Delta Z}$Phe[5]); 7.04 ppm (HNGly[1]); 7.14 ppm ($H^{\beta\Delta Z}$Phe[3]); 7.19–7.62 ppm (aromatic ring); 7.99 ppm ($H^{\delta\#}$pNA); 8.13 ppm (HNGly[2]); 8.22 ppm ($H^{\varepsilon\#}$pNA); 8.38 ppm (HNPhe[6]); 8.72 ppm (HNGly[4]); 9.66 ppm ($HN\Delta^{Z}$Phe[3]); 9.97 ppm ($HN\Delta^{Z}$Phe[5]); 10.06 ppm (HNpNA), ^{13}C NMR (DMSO, TMS): 28.0 ppm (CH3 Boc); 36.3 ppm (C^{β}Phe[6]); 42.4 ppm (C^{α}Gly[2]); 43.2 ppm (C^{α}Gly[1]); 55.9 ppm (C^{α}Phe[6]); 78.0 ppm (C^{γ}Boc), 119.1 ppm ($C^{\delta\#}$pNA); 124.8 ppm ($C^{\varepsilon\#}$pNA); 128.4 ppm ($C^{\beta\Delta Z}$Phe[5]); 128.2–137.7 ppm (aromatic ring); 128.8 ppm ($C^{\beta\Delta Z}$Phe[3]); 133.2 ppm ($C^{\alpha\Delta}$Phe[3]); 133.4 ppm ($C^{\alpha\Delta Z}$Phe[5]); 142.3 ppm (C^{ζ}pNA); 144.5 ppm (C^{γ}pNA); 155.7 ppm (COBoc); 164.4 ppm (C)OΔ^{Z}Phe[5]); 165.9 ppm (CO Δ^{Z}Phe[3]); 169.0 ppm (COGly[2]); 170.1 ppm (COGly[1]); 170.2 ppm (COGly[4]); 170.5 ppm (CPhe[6]). Elemental analysis for $C_{44}H_{46}N_8O_{10}$ calculated: 62.4% C, 5.47% H and 13.23% N, found: 60.50% C, 5.10% H and 12.73% N.

4.5. Peptide no. 3

^{1}H NMR (DMSO, TMS): 3.15 ppm (H^{β}Phe[6]); 3.61 ppm (H^{α}Gly[1]); 3.99 ppm (H^{α}Gly[4]); 4.05 ppm (H^{α}Gly[2]); 4.73 ppm (H^{α}Phe[6]); 6.97 ppm ($H^{\beta\Delta Z}$Phe[5]); 7.16 ppm ($H^{\beta\Delta Z}$Phe[3]); 7.24–7.43 ppm (aromatic ring); 7.99 ppm ($H^{\delta\#}$pNA); 8.07 ppm (HNGly[1]); 8.22 ppm ($H^{\varepsilon\#}$pNA); 8.41 ppm (HNPhe[6]), 8.81 ppm (HNGly[4]); 8.72 ppm (HNGly[2]); 9.80 ppm ($HN\Delta^{Z}$Phe[3]); 10.02 ppm ($HN\Delta^{Z}$Phe[5]); 10.08 ppm (HNpNA) ^{13}C NMR (DMSO, TMS): 36.3 ppm (C^{β}Phe[6]); 40.1 ppm (C^{α}Gly[1]); 42.3 ppm (C^{α}Gly[2]); 43.4 ppm (C^{α}Gly[4]); 55.9 ppm (C^{α}Phe[6]); 119.1 ppm ($C^{\delta\#}$pNA); 124.8 ppm ($C^{\varepsilon\#}$pNA); 128.2 ppm ($C^{\beta\Delta Z}$Phe[5]); 128.5–144.6 ppm (aromatic ring); 128.8 ppm ($C^{\beta\Delta Z}$Phe[3]); 133.2 ppm, 133.3 ppm ($C^{\alpha\Delta Z}$Phe[3], Δ^{Z}Phe[5]); 164.7 ppm (COΔ^{Z}Phe[5]); 165.9 ppm (COΔ^{Z}Phe[3]); 166.4 ppm (COGly[1]); 168.6 ppm (COGly[2]); 170.5 ppm (COGly[4]); 170.7 ppm (COPhe[6]).

4.6. Peptide no. 4

^{1}H NMR (DMSO, TMS): 1.36 ppm (CH3 Boc); 2.91 ppm ($H^{\beta 2}$Phe[6]); 3.14 ppm (H^{β}Phe[6]); 3.59 ppm (H^{α}Gly[1]); 3.95 ppm (H^{α}Gly[4]); 3.97 ppm (H^{α}Gly[2]); 4.76 ppm (H^{α}Phe[6]); 6.54 ppm ($H^{\beta\Delta E}$Phe[5]); 6.58 ppm ($H^{\beta\Delta E}$Phe[3]); 6.99–7.45 ppm (aromatic ring); 7.03 ppm (HNGly[1]); 7.98 ppm ($H^{\delta\#}$pNA); 8.19 ppm ($H^{\varepsilon\#}$pNA); 8.21 ppm (HNGly[2]); 8.50 ppm (HNGly[4]); 8.75 ppm (HNPhe[6]); 9.79 ppm ($HN\Delta^{E}$Phe[3]); 9.94 ppm ($HN\Delta^{E}$Phe[5]); 10.17 ppm (HNpNA), ^{13}C NMR (DMSO, TMS): 28.1 ppm (CH3 Boc); 36.4 ppm (C^{β}Phe[6]); 42.5 ppm (C^{α}Gly[4]); 42.6 ppm (C^{α}Gly[2]); 43.2 ppm (C^{α}Gly[1]); 55.2 ppm (C^{α}Phe[6]); 78.2 ppm (C^{γ}Boc); 118.21 ppm ($C^{\beta\Delta E}$Phe[3]); 118.22 ppm ($C^{\beta\Delta E}$Phe[5]); 119.2 ppm ($C^{\delta\#}$pNA); 124.8 ppm ($C^{\varepsilon\#}$pNA); 128.4–144.7 ppm (aromatic ring); 131.2 ppm, 131.4 ppm ($C^{\alpha\Delta E}$Phe[3], Δ^{E}Phe[5]); 155.9 ppm (CO Boc); 164.5 ppm (CO Δ^{E}Phe[3]); 164.8 ppm (COΔ^{E}Phe[5]); 165.3 ppm (COPhe[6]); 168.4 ppm (COGly[4]); 170.1 ppm (COGly[2]); 170.4 ppm (COGly[1]); Elemental analysis for $C_{44}H_{46}N_8O_{10}$ calculated: 62.4% C, 5.47% H and 13.23% N, found: 59.72% C, 5.39% H and 12.79% N.

4.7. Peptide no. 5

^{1}H NMR (DMSO, TMS): 1.37 ppm (CH3 Boc); 2.75 ppm ($H^{\beta 1}$Phe[3]); 2.77 ppm ($H^{\beta\#}$Phe[5]); 3.01 ppm ($H^{\beta 1}$Phe[6]); 3.02 ppm ($H^{\beta 2}$Phe[3]); 3.14 ppm ($H^{\beta 2}$Phe[6]); 3.55 ppm (H^{α}Gly[1]); 3.74 ppm (H^{α}Gly[2]); 3.77 ppm (H^{α}Gly[4]); 4.50 ppm (H^{α}Phe[3]); 4.55 ppm (H^{α}Phe[5]); 4.70 ppm (H^{α}Phe[6]); 7.02 ppm (HNGly[1]); 7.1–8.26 ppm (aromatic ring); 7.89 ppm ($H^{\delta\#}$pNA); 8.24 ppm ($H^{\varepsilon\#}$pNA); 7.92 ppm (HNGly[2]); 8.12 ppm (HNPhe[6]); 8.19 ppm (HNGly[2]); 8.32 ppm (HNGly[4]); 8.61 ppm (HNPhe[6]);

10.76 ppm (HNpNA), ^{13}C NMR (DMSO, TMS): 28.1 ppm (CH3 Boc); 37.3 ppm (C^{β}Phe[6]); 37.45 ppm (C^{β}Phe[5]); 37.48 ppm (C^{β}Phe[3]); 41.80 ppm (C^{α}Gly[4]); 41.81 ppm (C^{α}Gly[2]); 43.1 ppm (C^{α}Gly[1]); 53 93 ppm (C^{α}Phe[3]); 53 94 ppm (C^{α}Phe[5]); 55.3 ppm (C^{α}Phe[6]); 78.1 ppm (C^{γ}Boc); 118.8 ppm ($C^{\delta\#}$pNA); 124.7 ppm ($C^{\varepsilon\#}$pNA); 126.3–129.2 ppm (aromatic ring); 137.2–137.9 ppm (aromatic ring);; 142.2 ppm (C^{ζ}pNA); 144.8 ppm (C^{γ}pNA); 155.7 ppm (COBoc); 168.3 ppm (COGly[4]); 168.4 ppm (COGly[2]); 169.6 ppm (COGly[1]); 170.9 ppm (COPhe[5]); 170.8 ppm, 171.0 ppm (COPhe[3], COPhe[6]).

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